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Insights into pathogenesis of five novel *GCK* mutations identified in Chinese MODY patients

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Abstract

Objective: Heterozygous inactivating mutations in *GCK* are associated with defects in pancreatic insulin secretion and/or hepatic glycogen synthesis leading to mild chronic hyperglycemia of maturity onset diabetes of young type 2 (MODY2). However, the effect of naturally occurring GCK mutations on the pathogenesis for MODY2 hyperglycemia remains largely unclear, especially in the Asian population. The aim of this study is to explore the potential pathogenicity of novel GCK mutations related to MODY2.

Methods: Genetic screening for GCK mutations from 96 classical MODY families was performed, and structure-function characterization and clinical profile of identified GCK mutations were conducted.

Results: Five novel (F195S, I211T, V222D, E236G and K458R) and five known (T49N, I159V, R186X, A188T and M381T) mutations were identified and co-segregated with hyperglycaemia in their pedigrees. R186X generates non-functional truncated form and V222D and E236G fully inactivate glucokinase due to severe structure disruptions. The other seven GCK mutations exhibited marked reductions in catalytic efficiency and thermo-stability; notably, the interaction with GKRP was significantly enhanced in I211T, I159V, T49N and K458R, reduced in F195S and M381T, and completely lost with A188T. 31% (17/55) of MODY2 patients showed signs of insulin resistance. Conventional hypoglycemia treatment did not improve the HbA1C in MODY2 patients when insulin resistance is not present.

Conclusions: Five novel GCK mutations have been identified in Chinese MODY. The defects in enzymatic activity and protein stability, together with alteration of GKRP binding on GCK mutants may synergistically contribute to the development of MODY2 hyperglycaemia. No treatment should be prescribed to MODY2 patients when insulin resistance is not present.

Keywords: Glucokinase (GCK); Mutation; MODY2; Glucokinase regulatory protein (GKRP)**;** Chinese

Abbreviations

GCK	Glucokinase
GKRP	Glucokinase regulatory protein
GSIS	Glucose-stimulated insulin secretion
G6P	Glucose-6-phosphate
GKAs	Glucokinase activators
MODY	Maturity-onset diabetes of the young
mGCK	mutant GCK
OHA	Oral hypoglycaemic agents
WT GCK	wild-type GCK
Y2H	Yeast two-hybrid

1. Introduction

Glucokinase (GCK), specifically expressed in both the pancreatic β cells and hepatocytes, catalyzes the conversion of glucose to glucose-6-phosphate (G6P) [1]. In pancreatic β cells, GCK acts as a glucose sensor that determines glucose utilization, which is necessary for glucose-stimulated insulin secretion [2]. In hepatocytes, GCK determines the rate of both glucose uptake and glycogen synthesis and is essential for the regulation of various glucose-responsive genes [3]. Heterozygous inactivating mutations in GCK gene are associated with defects in pancreatic insulin secretion and/or hepatic glycogen synthesis leading to mild chronic hyperglycemia of maturity-onset diabetes of the young type 2 (MODY2) or GCK-MODY [4]. MODY2 is characterized by mild, non-progressive hyperglycaemia from birth; these patients rarely present with diabetic complications and

usually do not require routine pharmacological treatment [5, 6]. The heterogeneity of MODY2 subtype distribution among different ethnic groups has also been implicated，wherein the prevalence of MODY2 in Caucasian MODY cases is as high as 32–63% [4, 7-10]. With the advancement in the understanding of MODY2 phenotypes [11] and molecular diagnosis techniques, more and more GCK mutations were detected in Asian MODY2 patients [12, 13].

Global homozygous GCK knockout mice result in perinatal death from severe diabetes [14]. Heterozygous null for GCK mice develop early mild onset diabetes, which recapitulate the GCK-MODY phenotype. These mice exhibit reduced islet glucokinase activity causing mildly elevated fasting blood glucose levels, and decreased glucose tolerance and abnormal liver glucose metabolism were observed during hyperglycemic clamp studies [14]. These rodent's studies further confirm that heterozygous inactivation of GCK gene would lead to deficiencies in both β cells and hepatocytes resulting in the hyperglycemia of MODY2.

The known crystal structures of human GCK and its binding proteins [15, 16] reveal several critical functional domains which include glucose binding sites that form the active center of GCK and allosteric activator sites to which pharmacological GKAs bind [15], as well as glucokinase regulatory protein (GKRP) binding domains [16]. GKRP, acting as a competitive inhibitor with respect to glucose, regulates GCK activity and determines the subcellular location of glucokinase in liver [17]. When bound to substrates (glucose and ATP), glucokinase switches from a super-open inactive form to a closed active form catalyzing the production of G6P [15]. Therefore, introducing mutations into the closed active structure molecular model of glucokinase may help with the understanding of the abnormal biochemistry of different GCK mutations that result in altered GCK activity.

In order to gain further insight into the pathogenesis of GCK mutations, we have performed genetic screening of 96 Chinese MODY families for identifying possible

additional novel GCK mutations. Then using an integrated approach i.e. pedigree, molecular modeling, enzymology, GKRP inhibition and clinical profiling, we conducted the structure – function characteristics investigation of these naturally occurring GCK mutations. To address the concern for necessity treatment of hyperglycemia in MODY2, we also carried out a pharmacogenomics study to investigate the effect of glycaemic control with or without anti-diabetic pharmacological treatment in MODY2 patients.

2. Materials and Methods

2.1. Subjects

Medical and family history questionnaires were completed by all participants, whose information was supplemented with information from the medical records. The American Diabetes Association criteria (2013) were used to diagnose diabetes, impaired fasting glucose (IFG), and impaired glucose tolerance (IGT). Written informed consent was obtained from all participants. This study was approved by the Shanghai Jiaotong University Affiliated Sixth People's Hospital Institutional Review Board.

96 MODY families included 424 affected subjects, wherein 96 MODY probands who fulfilled the classical MODY criteria (non-obesity, absence of autoantibodies, at least one patient with onset age <25 years, and a family history of diabetes for at least three consecutive generations) [18], but had mostly been misdiagnosed and mistreated as type 1 or type 2 diabetes by local hospitals, and their family members from 2014 to 2016, were referred to or recruited by the Shanghai Diabetes Institute, Department of Endocrinology & Metabolism, Shanghai Jiaotong University Affiliated Sixth People's Hospital for GCK genetic testing, counselling and standardized clinical and laboratory evaluation [19]. Additionally, we enrolled unrelated 200 non-diabetic individuals, 118 male and 82 females, age, 66.3±2.7 years with the following criteria: age>60 years; body mass index (BMI),

22.0±0.7kg/m^2, normal glucose tolerance, glycated haemoglobin (HbA1c) <5.6% (38 mmol/mol), and no family history of diabetes.

2.2. Identification of *GCK* mutations

The genomic DNA of the 96 MODY pedigrees was isolated from peripheral blood and used for screening GCK mutations by PCR–direct sequencing and multiplex ligation-dependent probe amplification (MLPA). Twelve exons of the human *GCK* expressed in pancreatic β-cells and hepatocytes (GenBank accession no.AH005826) were screened using previously described primer sequences [20]. The identified mutations were then tested for co-segregation with hyperglycaemia (diabetes, IFG or IGT) in other family members and the 200 non-diabetic controls of Han Chinese origin. The mutations and variants were numbered according to the Human Genome Variation Society http:// www.hgvs.org/.

2.3. Construction of human glucokinase molecular model

The crystal structure of human glucokinase has been determined by Kamata et al [15]. GCK mutants were constructed according to the active holo closed conformation (3VEY: ATP+GCK activator+glucose, Protein Data Bank database, http://www.rcsb.org/ pdb/explore. do?structureId=3VEY PubMed: 22298776). The mutations were analysed at the structural level by introducing the modifications into the 3VEY structure model. In addition, deleterious mutations were predicted using online prediction programs SIFT, PolyPhen-2, LRT, MutationTaster, FATHMM, and RadialSVM.

2.4. Production of recombinant GCK, and kinetic analysis and thermostability assays

His-tagged wild-type (WT) and GCK mutants (mGCKs), generated by the QuikChange Site-Directed Mutagenesis Kit and the appropriate primers (Supplemental Table 1) were inserted into the expression vector pET28a to express the recombinant GCK proteins. The GCK proteins were expressed in *Escherichia coli* Rosetta 2 (DE3) pLysS, purified and stored as described previously [21, 22]. GCK activity was measured spectrophotometrically using a

SynergyTM4 multimode microplate reader (BioTek, Vermont, USA) by quantifying NADPH, which is directly proportional to the concentration of glucose-6-phosphate (G6P) generated by GCK. The kinetic parameters of WT GCK and its mutants were measured and calculated using Sigmaplot 13.0 (Systat Software, San Jose, USA). The relative activity index and glucose concentration at the inflection point were calculated as previously described [21]. The thermal stability of GCKs was determined as described previously [22]. Eight proteins were pre-incubated at various temperatures, i.e., 35°C, 40°C, 45°C, 50°C, 55°C, 60°C and 65°C for 30 min in a reaction buffer without substrates, then ATP (5 mM) and glucose (200 mM) were added to the reaction mixtures and incubated for 10 min at 37°C; the product G6P was quantified. The G6P yields of inactivated enzymes were divided by that of unheated inactivated enzymes and used as the remaining relative activity. Furthermore, the heat-inactivation curves at 52.5°C were determined for wild type of GCK and its mutants.

2.5. Yeast two-hybrid (Y2H) system

mGCK-encoding cDNAs were inserted into pGBKT7 vector (Clontech) using KpnI and BamHI to generate pGBKT7-mGCK, and then transformed into *Saccharomyces cerevisiae* strain Y2HGold as the bait. WT GCK was used as the positive control. GKRP-encoding cDNA was cloned into pGADT7 vector (Clontech) using KpnI and BamHI to generate pGADT7-GKRP, and then *S. cerevisiae* strain Y187 was transformed with the constructs as the prey. Diploid *S. cerevisiae* strains were obtained using small-scale transformation, and then plated on SD/-Leu/-Trp Agar, SD/-Leu/-Trp/(DDO), and SD/-Ade/-His/-Leu/-Trp/ X-a-Gal/AbA (QDO/X/A) agar plates; the activity of the secreted α-galactosidase was subsequently measured according to Matchmaker Gold Y2H System user manual [23].

2.6. Pharmacogenomics study

We compared HbA1c, age at diagnosis, and BMI in 38 MODY2 patients without insulin resistance (HOMA-IR<2) from ten MODY2 families, who were receiving oral

hypoglycaemic agents (OHA, n=17), insulin (n=12), or no treatment (n=9). The OHA-treated patients at referral received anti-diabetic drugs with low dose that equate to 500 mg metformin or 0.5 mg glimepiride per day. In the insulin-treated patients, the median insulin dose was 0.2 U/kg/day (range, 0.1–0.4 U/kg/day).

2.7. Statistical analysis

All clinical and laboratory values are presented as means ± SEM or as medians (interquartile range) unless otherwise stated. Comparisons of the clinical and laboratory parameters between or among groups were performed using unpaired Student's *t*-tests, where data with skewed distribution were logarithmically transformed before analysis, or one-way ANOVA and Pearson χ^2 tests as appropriate. $P<0.05$ was considered to be significant. SPSS19.0 (SPSS Inc.) was used for data analysis and processing.

3. Results

3.1. Genetic and clinical characteristics

Ten GCK mutations were identified in ten unrelated MODY families, including five novel (F195S, I211T, V222D, E236G and K458R) and five previously reported mutations (T49N, I159V, R186X, A188T and M381T [4, 5, 24-26] (Fig. 1, Supplemental Fig. 1, Supplemental Table 2). The sequences were found to be evolutionally well-conserved across different mammal species, except that I159 was substituted with L159 in the rat, and these mutations were co-segregated with hyperglycaemia (diabetes, IGT, or IFG) in their respective pedigrees (Fig. 1). GCK mutations were not identified in the other probands or the 200 controls, suggesting that these mutations are not simple polymorphisms.

In the ten pedigrees, MODY2 subjects (n=55) had significantly elevated fasting plasma glucose (FPG), 2-h postprandial plasma glucose (2hPG) and HbA1c，decreased fasting insulin (FINS)/FPG and 2-h insulin (2hINS)/2hPG, compared to their unaffected relatives

(n=36) (all, p<0.001, Table 1). It was noteworthy that 31% (n=17) of MODY2 patients showed signs of insulin resistance with homeostatic assessment of insulin resistance (HOMA-IR) index ≥2.0，the HbA1c of which was significantly higher than those without insulin resistance（6.7±0.3 vs 6.4±0.2，p=0.038, Supplemental Table 3). The onset age and BMI in the former were also higher respectively than that in the latter, although no significant differences were observed (both p>0.05, Supplemental Table 3). None of these patients had any chronic diabetic complications.

3.2. mGCK model construction

To investigate whether the conformational alterations caused by mutations contribute to the change of protein function, mGCK molecular models were constructed. As shown in Fig. 2a, the location of the ten GCK mutations was modelled on the global landscape of the GCK protein structure. Then, the mutations were studied at the structural level by introducing the modifications into the 3VEY structure model (Fig. 2b-k). The V222D, E236G, and R186X mutations were predicted to result in the most serious damage to the spatial conformation (Fig. 2b-d). **V222D** – Valine 222 formed a large hydrophobic core of the large domain with the side chains of M224, A232, Y234, L386, V389, I390, M393, I404 and V406 residues (Fig. 2b). A negative-charge polar hydrophilic D222 residue replaced a non-polar hydrophobic V222 residue, severely destroying the stability of the hydrophobic core in the large domain. **E236G** – A non-polar hydrophobic and uncharged Glycine 236 substituted a negative-charge polar hydrophilic residue E236, causing the loss of the salt bridge–hydrogen bond network formed by E236 with Y234, E237, N240, and R392 (Fig. 2c). **R186X** - The R186X mutation generated a truncated protein lacking 280 amino acids, as only two glucose binding sites, i.e., Thr168 and Lys169 in the small domain were reserved (Fig. 2d); it could not bind to glucose and catalyse the reaction from glucose and ATP to G6P.

However, the other seven mutations i.e., T49N, I159V, A188T, F195S, I211T, M381T

and K458R resulted in different degrees of GCK instability through a change in the three-dimensional (3D) structure (Fig. 2e-k), thus leading to decreased enzyme activity. **T49N** - N49 formed a pair of new hydrogen bonds with the side chains of E48, but lost the hydrogen bond with R43 (Fig. 2e). **I159V** - I159V mutation resulted in shorter side chains, thereby weakening the hydrophobic interaction with surrounding residues (Fig. 2f). **A188T** -The CH3 group on the side chain of T188 embedded deeply inside may form a larger steric hindrance with surrounding residues (Fig. 2g). **F195S** - F195S mutation impairs local hydrophobic interaction formed with I130, L134, I189 and M197 (Fig. 2h). **I211T** - The I211T, a mutation located on allosteric activator site, reduces the hydrophobic interaction and encapsulates the OH groups of the T-side chains in the interior to form unsaturated hydrogen bonds with large dewatering free energy (Fig. 2i). **M381T -**The side chain OH groups of T381 forms hydrogen bonds with the main chain carbonyl O of R377, but it is difficult to offset the unfavorable dewatering free energy (Fig. 2j). **K458R** - K458 substituted by R458 may not affect the distribution of the surrounding charge and the solubility of protein due to the similar physical and chemical properties (Fig. 2k). Moreover, at least five of the six online prediction tools predicted that these ten mutations were deleterious (Supplemental Table 4) (24).

3.3 mGCK kinetic analysis and thermostability

To investigate how the GCK mutations affect GCK protein's function, we analysed GCK catalytic activity. Recombinant proteins of mGCK were expressed and purified (Fig. 3a), GCK activity and thermostability were determined. The activity of V222D, E236G and R186X were too low to generate clear products in the presence of 1 mg/mL protein, indicating that three mutations are almost completely inactive (Fig. 3b). All other mutations affect GCK activity with different severities. The GCK activities of these mutants compared to wild type GCK were decreased in the order of WT>M381T> K458R>I211T>

I159V>F195S>T49N>A188T (Fig. 3b), which is consistent with their catalytic efficiency for glucose (Kcat/S0.5) (Table 2). Thermostability is a critical criterion of protein which can be used to evaluate protein structure stability. Compared with WT GCK, all seven mGCKs shows decreased thermostability, especially M381T. The GCK thermostabilities were in the order of WT>I159V> K458R>F195S>A188T>I211T>T49N>M381T. All mGCKs lost >50% activity at 49°C, the temperature at which WT GCK lost 50% activity (Fig. 3c). Compared with the half-life of WT (7.2 min) at 52.5°C, the mGCKs have shorter ones (Fig. 3d). All seven mutants exhibit reduced GCK activities and thermostability. It is noteworthy that V222D, E236G and R186X three mutants are completely inactive and dynamic parameters and thermostability analysis of them was not applicable.

3.4. GCK mutations altered interactions with GKRP

Since GCK is also regulated through protein-protein interactions by the glucokinase regulatory protein (GKRP), which inhibits the enzyme and also induces its nuclear retention in hepatocytes, we investigated that whether these mutation of GCK affect interaction of mGCKs with GKRP using yeast two hybrid (Y2H) system. As showed in Fig. 4, the interaction strength was quantitatively determined via assaying the α-galactosidase activity, in comparison to WT GCK, the I211T, I159V, T49N and K458R mutations exhibited enhanced inhibition by GKRP, while F195S and M381T showed decreased interactions with GKRP as reflected by the activity of α-galactosidase ($p<0.05$ or $p<0.001$), in which the I211T revealed the strongest inhibition (Fig. 4). However, the activity of α-galactosidase expressed by the reporter gene *GLA* was not detected from the *S. cerevisiae* expressing the A188T (Fig. 4) and R186X mutants (data not shown). Yeast auxotrophic selection confirmed this result, as the diploid *S. cerevisiae* strain expressing the A188T mutant and GKRP could not survive under this condition (data not shown).

3.5. Pharmacological treatment did not affect HbA1c in MODY2 patients without

insulin resistance

To assess whether patients with MODY2 need hypoglycaemic therapy, the effects of pharmacological treatment and the absence thereof on their glycaemic control were evaluated. Of the 38 MODY2 patients without insulin resistance, there was no significant difference in HbA1c between patients on pharmacological therapy (n=29) and those without (n=9): OHA+insulin vs. no treatment: 6.4±0.1 vs. 6.3±0.3 (p=0.618, Fig. 5), indicating pharmacological treatment for these patients did not alter glycaemic control. The patients on OHA treatment were older at onset age than the patients on insulin or without medication: OHA vs. insulin vs. no treatment, 24 (21.5, 34.0) vs. 14.5(2.6, 28.0) vs. 12 (2.2, 24.0) years (p=0.005).

4. Discussion

GCK phosphorylates glucose to G6P and plays a major role in regulating glucose metabolism in pancreatic β-cells and hepatocytes [1]. The glucokinase gene consists of 12 exons and encodes a 465-amino-acid protein [27] with three tissue-specific isoforms due to alternative splicing at exon 1 [28]. The ten GCK mutations identified in this study are localized to exons 2–10, which are common exons for both the pancreatic and hepatic GCK isoforms. Therefore, these mutations would cause GCK dysfunctions in both cell types and result in fasting and postprandial hyperglycaemia in these MODY2 patients (Table 1). Decreased FINS/FPG and 2hINS/2hPG in MODY2 patients (Table 1) demonstrate the defects in insulin secretion and agree with the observation in Caucasians and Japanese MODY2 patients as reported previously [4, 12]. Interestingly, 31% (17/55) of MODY2 patients showed signs of insulin resistance (HOMA-IR≥2) in this study, which was also seen in 25% of Japanese MODY2 patients [12], demonstrating that familial insulin resistance is more common in Asian people [29, 30]. Nevertheless, despite the uneven enzyme activity exhibited in these ten GCK

mutants (Table 2), no differences in MODY2 phenotypes were observed, thus we cannot establish a clear relationship between the severity of the biochemical defect and the actual diagnosis and treatment. These data support the hypothesis that similar phenotype of MODY2 patients resulting from heterozygous mutations in GCK could be explained by the fact that one adapted wild-type allele could compensate for the enzyme defects caused by the mutant allele [21].

4.1. Both complete and partial inactivation of GCK led to MODY2

Ten mutations were identified in 96 MODY pedigrees in mainland China, five of which are reported for the first time (F195S, I211T, V222D, E236G and K458R). We found close association between structural alterations of these mutants predicted by molecular modeling and their enzyme activities. Based on the GCK enzymatic activity, the identified mutations can be classified into two main categories: completely inactive mutations (V222D, E236G and R186X) and partially inactive mutations (T49N, I159V, F195S, I211T, M381T, K458R and A188T).

R186X mutation generates a truncated form shorter than E440X that cuts the only vital C-terminal end of the GCK peptide and inactivates the cytoplasmic enzymatic activity of the protein [31], thus R186X was predicted to be a completed loss of function of GCK. As expected, we observed that R186X mutant does not have any GCK enzymatic activity. V222D mutation introduces a net negative charge into a hydrophobic environment, which destroyed the large hydrophobic core formed with surrounding nine residues in the large domain (Fig. 2b). E236G mutation disrupted the perfect salt bridge hydrogen bond network formed with Y234, E237, N240 and R392 residues (Fig. 2c). V222D and E236G caused nearly complete loss of enzyme activity, similar to R186X which lost four glucose binding sites of GCK (Fig. 2d, Fig. 3b). Similarly, Gly162Asp introduced a net negative charge into a hydrophobic core [32] and Arg392Ser destroyed the salt bridge bond [33], which have been

reported to lead to the development of MODY2. Therefore, the associated kinetic parameters and thermo-stabilities of V222D and E236G could not be accurately determined due to the full loss of enzyme activity, similar to that of R186X (Table 2). Although R186X was reported to be a mutation resulting in a truncated protein, no known studies have thoroughly analysed the structure and the G-6-P generation activity of this fully inactivated protein previously.

Enzymatic activity studies indicate that partial inactive mutations (I211T, I159V, T49N, K458R, F195S, M381T and A188T) exhibited a reduction in relative activity index and protein instability (Table 2, Fig. 3c, 3d). Notably, the interaction with GKRP was significantly enhanced for the four mutants (I211T, I159V, T49N and K458R), decreased in F195S and M381T, and totally lost with A188T (Fig. 4) compared to that of the wild-type ($p<0.05$ or $p<0.001$). Among them, M381T shows the mildest decrease in catalytic efficiency (Kcat1/S0.5) and the strongest effect on protein thermo-stability (Table 2, Fig. 3c, 3d), while A188T has the lowest enzyme activity and lost the interaction with GKRP (Table 2, Fig. 4). Enhanced GKRP inhibition for the four mutations may be the result of an increase in the binding affinity between the super-open form of GCK and GKRP, which may further decrease enzymatic activity and cause mis-localization of the protein. In addition, decreased or lost interactions between GKRP and F195S, M381T or A188T may mean that defects in the regulation of GCK by GKRP lead to catalytic instability [34] or hepatic nuclear reserves of these mutant enzymes were markedly reduced [35], which consequently hinders the clearance of postprandial glucose by the liver, thus contributing to the hyperglycemia in this MODY patients. The lack of GKRP inhibition of A188T may be explained by the fact that A188 is adjacent to R186 which reinforce the hydrophobic contact between the small domain residues of GCK and GKRP [16], while A188 is spatially close to His-141 to Leu-144, the binding sites of GCK with GKRP [36], the mutational T188 may change the

spatial conformation , prevents R186 function and/or covers the GKRP binding site. However, the mechanism of how mutations affect the interaction between GCK and GKRP will be confirmed with crystal structure analysis of the mutational GCK-GKRP complex and the hepatocyte localization experiment in vitro. Thus, these findings may offer new evidence to support the notion that the disturbance of GCK-GKRP may be account for some of MODY2 underlying mechanism for postprandial hyperglycaemia (Table 1) resulting from decreased hepatic glycogen accumulation and augmented hepatic gluconeogenesis observed in glucokinase-deficient patients [37].

4.2. Possible alternative strategy for MODY2

Our study found that 69% (38/55) of MODY2 patients showed no insulin resistance, wherein there was no difference in HbA1c in the patients on pharmacological regimes (n = 29) and those without (n = 9) (Fig.5). Therefore, our results provided an evidence for MODY2 patients who do not require hypoglycaemic therapy [6], however, no-treatment should only be limited to those patients without insulin resistance. The lack of response to routine hypoglycaemic medicine (insulin or OHA) may support a possibility i.e. the presence of a glucose sensing defect in patients with MODY2 results in a glucose level that is regulated at its raised level [38]. Thus, MODY2 patients and their family members could halt their conventional anti-diabetic treatment to avoid medication adverse effects, which represents a special example of pharmacogenomics in hypoglycaemic therapy.

The HbA1c levels of patients with insulin resistance were significantly elevated as compared with those of patients without insulin resistance（6.7±0.3 vs 6.4±0.2，p=0.038, Supplemental Table 3）. Therefore, these patients should be treated with OHA such as metformin to improve their insulin sensitivity. Our results demonstrated a new insight that patients with MODY2 without insulin resistance do not require hypoglycaemic therapy. In addition, despite mild hyperglycaemia, the elevation of HbA1c and the generation of

advanced glycation end products (AGE) that induces various deleterious biological effects [39] may be responsible for the development of diabetic microvascular complication in 4-6% of MODY2 patients [4]. Therefore, it is essential to develop new hypoglycaemic drugs with GKA and GCK-GKRP disruptor targeting to elevate GCK activity, while single or combined application of them may be expected to improve hyperglycaemia of MODY2 patients.

4.3. Misdiagnosis of MODY2

In addition, the patients on OHA treatment were older at onset age than the patients on insulin or without medication: 24 (21.5, 34.0) vs. 14.5(2.6, 28.0) vs. 12 (2.2, 24.0) years ($p=0.005$), suggesting that the onset in children or adolescents were usually misdiagnosed as type 1 diabetes, thus insulin was prescribed; those with onset in later life were easily misdiagnosed as type 2 diabetes, and were treated with OHAs. Our results suggested that, without genetic diagnosis, the clinical misdiagnosis rate of MODY2 was 85.4% (47/55) in China; moreover, 58.2% (32/55) of MODY2 patients received incorrect treatment. Similarly, the US SEARCH for Diabetes in the Youth study reported that most MODY cases were misdiagnosed as type 1 (36%) or type 2 diabetes (51%), wherein 100% of MODY2 patients were clinically misdiagnosed and 50% were receiving the wrong treatment [40]. The key driver for precision diabetes is that genetic aetiology strongly influences treatment choice and the clinical course [19, 41]. Therefore, genetic diagnose of MODY and differentiating MODY from type 1 and type 2 diabetes is crucial to enable the correct classification of diabetes and precise individual treatment.

4.4. Estimated more than 1.2 million MODY patients in China

The most recent diabetes epidemiology study in China in 2017 reported that the estimated overall prevalence of diabetes and pre-diabetes was 10.9% and 35.7%, or 119 million and 388 million individuals, respectively [42]. In case that MODY constitutes 1–5% of diabetes cases

[18], there may be at least 1.2 to 6.0 million MODY patients in China, which is around 100-fold of the number of British Caucasian MODY patients (26,000) [9]. GCK-MODY may be one of the two frequent subtypes among the 13 MODY subtypes discovered in the Chinese population so far [7, 13, 19, 43, 44], which is supported by this study that observed a 10.4% frequency of MODY2 occurrence (10 out of 96 families). Implementation of genetic testing of MODY2 and personalization of their hypoglycaemic medication may have great benefit to these MODY2 patients.

One of interesting findings of the present study is that MODY2 occurrence frequency is much higher than previously reported. According to conventional MODY criteria [18], 96 MODY probands and their family members who had been misdiagnosed as type 1 or type 2 diabetes were recruited. Among them, 10 GCK mutations, i.e., 10.4% frequency of MODY2 were observed. This is much higher than the rate of GCK mutation (1%) found in the Hong Kong study using minimum MODY criteria [7]. However, the present study also had some limitations. The result of this cross-sectional study indicates that MODY2 patients without insulin resistance should not be prescribed any treatment, however no follow-up study has been performed to confirm this. Therefore, a drug withdrawal follow-up study combining genetic tests with a larger number of patients should be conducted. Furthermore, to investigate the more accurate mutation spectrum and prevalence of MODY2 among Chinese patients with MODY, additional studies should expand the number of MODY pedigrees.

In summary, five new and five known mutations of GCK were identified from 96 MODY pedigrees. Structural alteration of these mutations led to defects in enzymatic activity and protein stability. Together with the abnormality of GKRP regulations on GCK mutants, these alterations may synergistically result in the development of hyperglycaemia of MODY2 in Chinese. This study proposes that MODY2 patients and their family members without insulin resistance could halt their conventional anti-diabetic treatment to avoid unnecessary

medication adverse effects. Since MODY2 patients without insulin resistance lack response to conventional hypoglycaemic agents, the development and use of new medication targeting to increased GCK activity, i.e. GKA and/or GCK-GKRP disruptors, could be a possible new therapeutic approach to treat MODY2 patients.

Author Contributions

LL designed the experiments; LL, YL, XL, and CC were responsible for the coordination of the project; LL, YL, YW, CC, XL and MJ contributed to drafting and revising the manuscript; LL, YL and XG contributed to the construction of molecular models and bioinformatics study. XL, ML, CC and LL contributed to kinetic analysis and thermostability study, GCK-GKRP interaction and data analysis. XG, YW, MJ and DY contributed to statistics and interpretation of data. ML, JY, JZ, YC, RZ, YJ, WZ, ML, LZ and TZ contributed to the recruitment of MODY families, blood samples collection, data acquisition, genotyping, and genotype-phenotype analysis. All authors have read and approved the final version of the manuscript.

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Conflict of Interest

The authors declare no conflict of interest.

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Table legends

***Table 1*: Clinical and biochemical parameters of ten MODY2 family members**

Data are the means±SEM, medians (interquartile range), or n (%).

[a] GCK mutation-negative, normal glucose tolerance first-degree relatives.

M=male; BMI=body mass index; FPG=fasting plasma glucose; 2hPG=2-h postprandial plasma glucose; FINS=fasting plasma insulin; 2hINS=2-h postprandial plasma insulin.

***Table 2:* Kinetic parameters of WT and mGCKs**

As the activity of V222D, E236G, and R186X were so low such that they did generate clear products in the presence of 1 mg/mL protein, the calculating dynamics parameters could not be calculated. Data are the means±SEM of three separate enzyme expressions. *h*=Hill coefficient; k_{cat}=GCK catalytic constant; S0.5=affinity constant for glucose; K_m=affinity constant for ATP; nd=not detected. vs. wild-type, ***p<0.001, **p<0.01, *p<0.05.

Figure legends

***Figure 1:* Pedigrees, genotypes, and clinical characteristics of ten MODY2 families**

(a-j): Ten family trees: black circles and squares, participants diagnosed with MODY2; half-filled black circles and squares, IGT or IFG; white circles and squares, normal glucose tolerance (NGT); red arrows, probands for the ten families. Numbers under the symbols are the family members' identification numbers, followed by the genotype of mutation, then age at diagnosis of diabetes and age at examination, followed by treatment for diabetes before

genetic diagnosis. nd=not detected; OHA=oral hypoglycaemic agents; N=normal allele; m=mutant allele.

Figure 2: **Model construction and free energy calculation of WT and mGCKs**

(a) Location of ten mutations in the 3D model of human β-cell GCK. The GCK structure in the active closed form (3VEY) is shown: purple, small domain; blue, large domains; grey, connecting region; yellow spheres, glucose; multi-colored chain, ATP. Six glucose binding sites, i.e., T168, K169, E256, E290, N204, and D205, are marked with red in the GCK molecular chain. Deep pink or yellow chains, mutation sites. **(b–k)** Effects of the ten mutations on the GCK structure model. Green spheres, WT GCK; red spheres, mGCKs. Interacting residues that formed the hydrophobic cores with WT residues at the mutated sites are marked in deep yellow in the GCK molecular chain. Yellow spheres, glucose; multi-colored chain, ATP.

Figure 3**: Purification, activity, and thermostability of mGCKs**

(a) Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of purified WT GCK and mGCK proteins. Proteins (2 μg per lane) were loaded onto 15% SDS-PAGE to confirm their purity. **(b)** Comparison of mGCK activity. The G6P yield was analysed under different enzyme concentrations (n=3). **(c)** Remaining mGCK activity after incubation at different temperature. **(d)** Time course of GCK heat inactivation at 52.5°C.

Figure 4**: Y2H quantification of the interaction between mGCK and GKRP**

Quantification of secreted α-galactosidase activity from the culture supernatant of Y2H Gold yeast colonies expressing mGCK and human GKRP. Bars and error bars, means±SEM. *p<0.05, ***p<0.001.

Figure 5: **Comparison of HbA1c levels among MODY2 patients with or without pharmaceutical treatment in a cross-sectional study**

HbA1c levels of MODY2 patients treated with OHAs (n=17), insulin (n=12), or no treatment (n=9) before genetic testing. Bars and error bars, means±SEM.

***Table 1*: Clinical and biochemical parameters of ten MODY2 family members**

	MODY2 patients (n=55)	Unaffected relatives [a] (n=36)	p-value
Sex (M)	34 (61.8%)	16 (44.4%)	0.133
Age (years)	35.0（12.0, 60.0）	37.5（30.1, 57.8）	0.491
Age at diagnosis of diabetes (years)	23.0（12.0, 30.0）	-	
Diabetes duration (years)	14.0（2.5, 27.0）	-	
BMI (kg/m^2)	21.3±2.5	22.1±1.9	0.391
HbA1c (%)	6.5±0.2	5.1±0.1	< 0.001
FPG (mmol/L)	7.2±0.3	4.7±0.2	< 0.001
2hPG (mmol/L)	10.0±0.8	5.8±0.4	< 0.001
FINS (mU/L)	4.7 (2.9, 6.9)	5.3（4.6, 6.0）	0.107
2hINS (mU/L)	22.9 (14.2, 35.3)	30.0（25.1, 37.2）	0.188
FINS/FPG (mU/mmol)	0.7 (0.4, 1.0)	1.2（1.0, 1.3）	< 0.001
2hINS/2hPG (mU/mmol)	2.4 (1.4, 3.7)	4.9（4.2, 6.8）	< 0.001

Data are the means±SEM, medians (interquartile range), or n (%).

[a] GCK mutation-negative, normal glucose tolerance first-degree relatives.

M=male; BMI=body mass index; FPG=fasting plasma glucose; 2hPG=2-h postprandial plasma glucose; FINS=fasting plasma insulin; 2hINS=2-h postprandial plasma insulin.

Table 2: **Kinetic parameters of WT and mGCKs**

Mutation	Yield (mg/l)	S0.5 (mM)	Hill coefficient (*h*)	Inflection point (mM)	ATP, K_m (mM)	Glucose, k_{cat1} (s^{-1})	ATP, k_{cat2} (s^{-1})	Activity index	Relative activity index	k_{cat1}/S0.5 (s^{-1}/mM)
WT	24.97±0.11	7.67±0.16	1.75±0.04	3.65±0.19	0.50±0.01	11.47±0.67	16.96±0.18	0.26±0.04	1.00	1.50±0.12
V222D	6.90±0.05***	nd	nd	nd	nd	nd	nd	nd	nd	nd
E236G	16.30±0.10***	nd	nd	nd	nd	nd	nd	nd	nd	nd
R186X	20.03±0.11***	nd	nd	nd	nd	nd	nd	nd	nd	nd
T49N	21.0±0.13***	31.13±0.70***	1.58±0.06	11.96±0.73***	0.44±0.00***	4.48±0.16***	15.48±0.05***	0.02±0.00***	0.08	0.14±0.01***
I159V	22.1±0.15***	35.85±1.39***	1.37±0.01***	9.18±0.41***	0.50±0.01	10.31±0.28	13.84±0.16***	0.06±0.01**	0.23	0.29±0.01***
F195S	21.57±0.11***	20.04±0.58***	1.78±0.01	9.81±0.26***	0.63±0.01***	4.50±0.05***	13.71±0.13***	0.02±0.00***	0.08	0.22±0.01***
I211T	22.4±0.08**	13.94±0.14***	1.56±0.04*	5.20±0.29*	0.40±0.01***	8.98±0.35*	10.34±0.12***	0.13±0.02*	0.5	0.64±0.02***
M381T	24.13±0.13	11.20±0.43**	1.64±0.04	4.72±0.13**	0.49±0.01	11.52±0.21	16.36±0.11*	0.18±0.01*	0.69	1.03±0.03**
K458R	23.6±0.08**	11.02±0.05***	1.82±0.01*	5.61±0.06***	0.61±0.01**	10.77±0.08	16.01±0.18*	0.10±0.01**	0.38	0.98±0.01**
A188T	23.17±0.11***	51.70±0.48***	1.34±0.02***	12.40±0.56***	0.35±0.00***	3.17±0.09***	12.43±0.06***	0.01±0.00***	0.04	0.06±0.00***

As the activity of V222D, E236G, and R186X were so low such that they did generate clear products in the presence of 1 mg/mL protein, the calculating dynamics parameters could not be calculated. Data are the means±SEM of three separate enzyme expressions. *h*=Hill coefficient; k_{cat}=GCK catalytic constant; S0.5=affinity constant for glucose; K_m=affinity constant for ATP; nd=not detected. vs. wild-type, ***p<0.001,

**p<0.01, *p<0.05.

Highlights

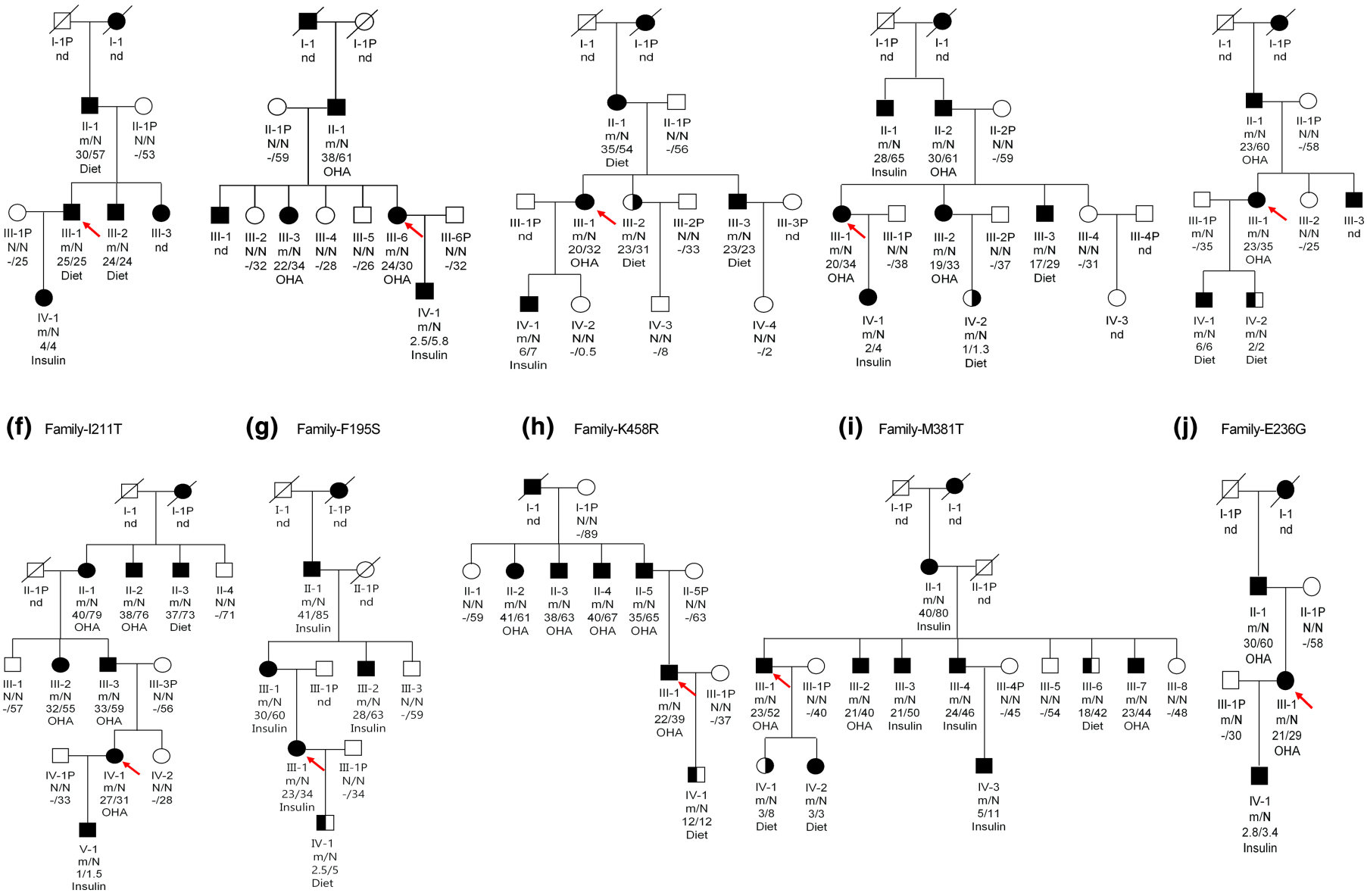
1) Five novel and five known mutations were identified in MODY pedigrees.

2) MODY2 accounts for 10.4% of classical MODY cases in Chinese in this study.

3) Defects in enzymatic activity, stability and GKRP interaction may co-induce MODY2.

4) 31% of MODY2 patients showed signs of insulin resistance

5) No treatment should be prescribed to MODY2 patients without insulin resistance.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I-1 nd
II-1 m/N 30/57 Diet
II-1P N/N -/53
III-1P N/N -/25
III-1 m/N 25/25 Diet
III-2 m/N 24/24 Diet
III-3 nd
IV-1 m/N 4/4 Insulin
(b) Family-R186X
I-1 nd
I-1P nd
II-1P N/N -/59
II-1 m/N 38/61 OHA
III-1 nd
III-2 N/N -/32
III-3 m/N 22/34 OHA
III-4 N/N -/28
III-5 N/N -/26
III-6 m/N 24/30 OHA
III-6P N/N -/32
IV-1 m/N 2.5/5.8 Insulin
(c) Family-T49N
I-1 nd
I-1P nd
II-1 m/N 35/54 Diet
II-1P N/N -/56
III-1P nd
III-1 m/N 20/32 OHA
III-2 m/N 23/31 Diet
III-2P N/N -/33
III-3 m/N 23/23 Diet
III-3P nd
IV-1 m/N 6/7 Insulin
IV-2 N/N -/0.5
IV-3 N/N -/8
IV-4 N/N -/2
(d) Family-V222D
I-1P nd
I-1 nd
II-1 m/N 28/65 Insulin
II-2 m/N 30/61 OHA
II-2P N/N -/59
III-1 m/N 20/34 OHA
III-1P N/N -/38
III-2 m/N 19/33 OHA
III-2P N/N -/37
III-3 m/N 17/29 Diet
III-4 N/N -/31
III-4P nd
IV-1 m/N 2/4 Insulin
IV-2 m/N 1/1.3 Diet
IV-3 nd
(e) Family-I159V
I-1 nd
I-1P nd
II-1 m/N 23/60 OHA
II-1P N/N -/58
III-1P m/N -/35
III-1 m/N 23/35 OHA
III-2 N/N -/25
III-3 nd
IV-1 m/N 6/6 Diet
IV-2 m/N 2/2 Diet
(f) Family-I211T
I-1 nd
I-1P nd
II-1P nd
II-1 m/N 40/79 OHA
II-2 m/N 38/76 OHA
II-3 m/N 37/73 Diet
II-4 N/N -/71
III-1 N/N -/57
III-2 m/N 32/55 OHA
III-3 m/N 33/59 OHA
III-3P N/N -/56
IV-1P N/N -/33
IV-1 m/N 27/31 OHA
IV-2 N/N -/28
V-1 m/N 1/1.5 Insulin
(g) Family-F195S
I-1 nd
I-1P nd
II-1 m/N 41/85 Insulin
II-1P nd
III-1 m/N 30/60 Insulin
III-1P nd
III-2 m/N 28/63 Insulin
III-3 N/N -/59
III-1 m/N 23/34 Insulin
III-1P N/N -/34
IV-1 m/N 2.5/5 Diet
(h) Family-K458R
I-1 nd
I-1P N/N -/89
II-1 N/N -/59
II-2 m/N 41/61 OHA
II-3 m/N 38/63 OHA
II-4 m/N 40/67 OHA
II-5 m/N 35/65 OHA
II-5P N/N -/63
III-1 m/N 22/39 OHA
III-1P N/N -/37
IV-1 m/N 12/12 Diet
(i) Family-M381T
I-1P nd
I-1 nd
II-1 m/N 40/80 Insulin
II-1P nd
III-1 m/N 23/52 OHA
III-1P N/N -/40
III-2 m/N 21/40 OHA
III-3 m/N 21/50 Insulin
III-4 m/N 24/46 Insulin
III-4P N/N -/45
III-5 N/N -/54
III-6 m/N 18/42 Diet
III-7 m/N 23/44 OHA
III-8 N/N -/48
IV-1 m/N 3/8 Diet
IV-2 m/N 3/3 Diet
IV-3 m/N 5/11 Insulin
(j) Family-E236G
I-1P nd
I-1 nd
II-1 m/N 30/60 OHA
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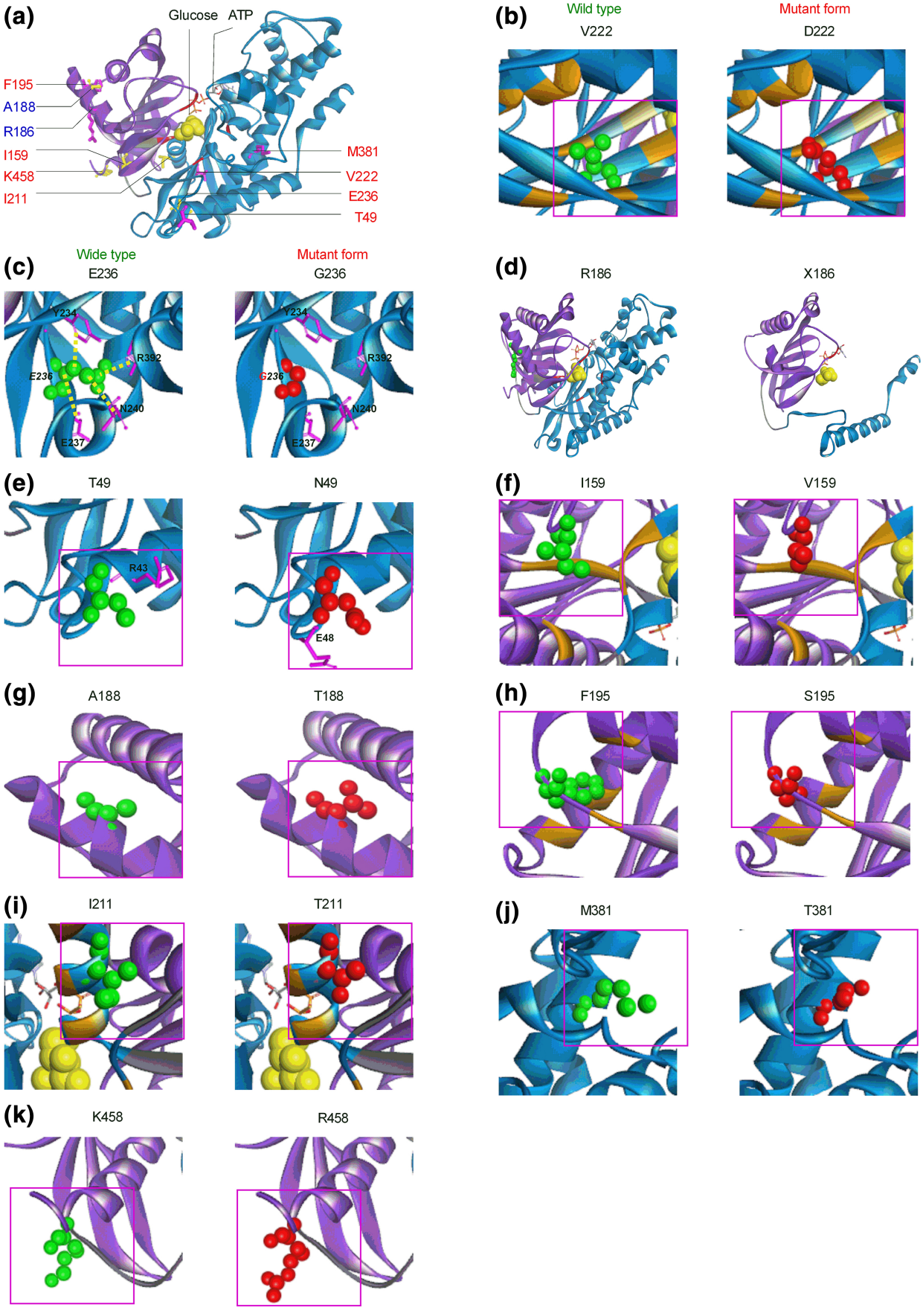
III-1P m/N -/30
III-1 m/N 21/29 OHA
IV-1 m/N 2.8/3.4 Insulin

Figure 1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Glucose
ATP
F195
A188
R186
I159
K458
I211
M381
V222
E236
T49
(b)
Wild type
V222
Mutant form
D222
(c)
Wide type
E236
Mutant form
G236
Y234
R392
E236
N240
E237
Y234
R392
G236
N240
E237
(d)
R186
X186
(e)
T49
R43
N49
E48
(f)
I159
V159
(g)
A188
T188
(h)
F195
S195
(i)
I211
T211
(j)
M381
T381
(k)
K458
R458

Figure 2

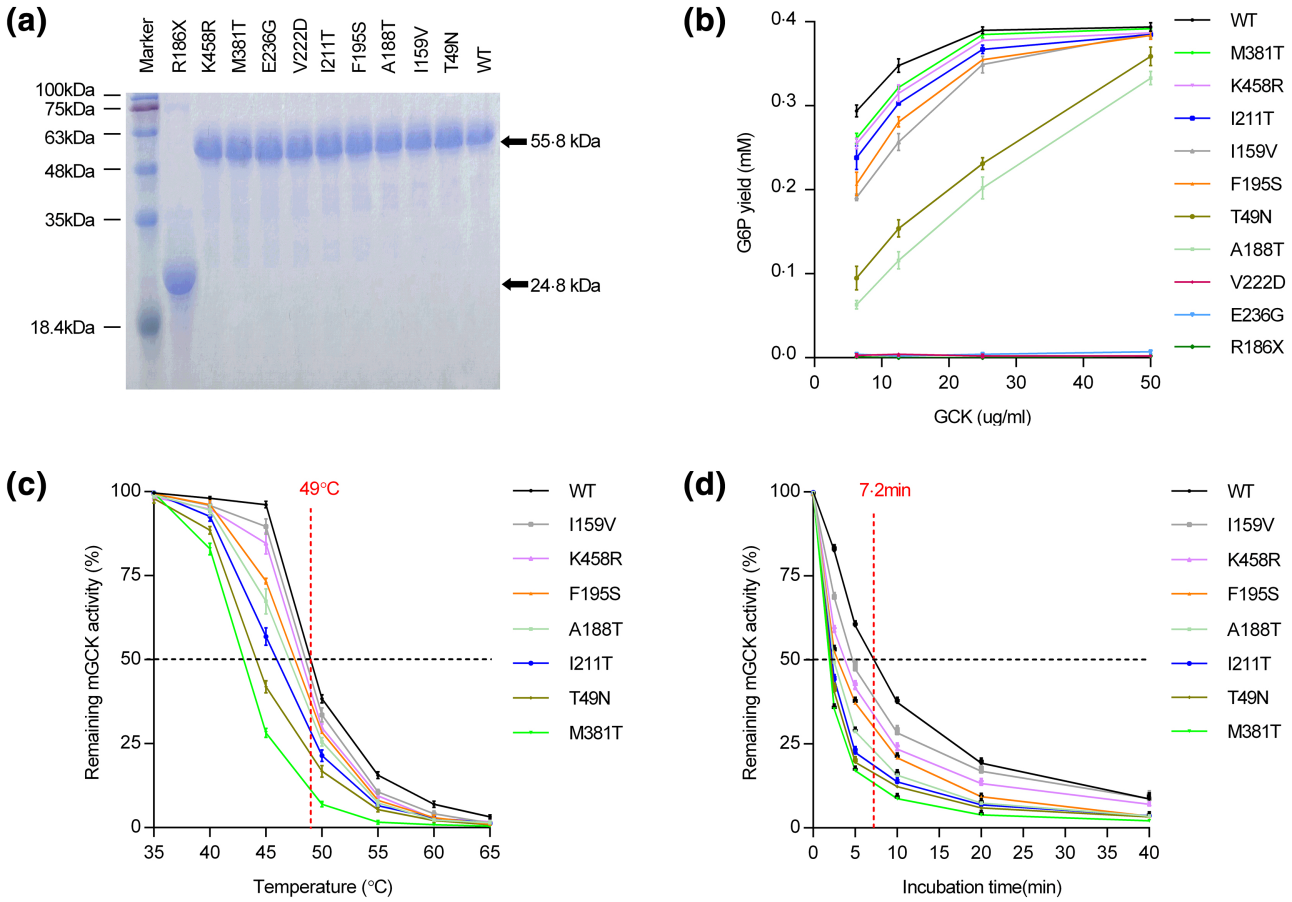
(a)
Marker
R186X
K458R
M381T
E236G
V222D
I211T
F195S
A188T
I159V
T49N
WT
100kDa
75kDa
63kDa
48kDa
35kDa
18.4kDa
55·8 kDa
24·8 kDa
(b)
G6P yield (mM)
GCK (ug/ml)
WT
M381T
K458R
I211T
I159V
F195S
T49N
A188T
V222D
E236G
R186X
(c)
49°C
Remaining mGCK activity (%)
Temperature (°C)
WT
I159V
K458R
F195S
A188T
I211T
T49N
M381T
(d)
7·2min
Remaining mGCK activity (%)
Incubation time(min)
WT
I159V
K458R
F195S
A188T
I211T
T49N
M381T

Figure 3

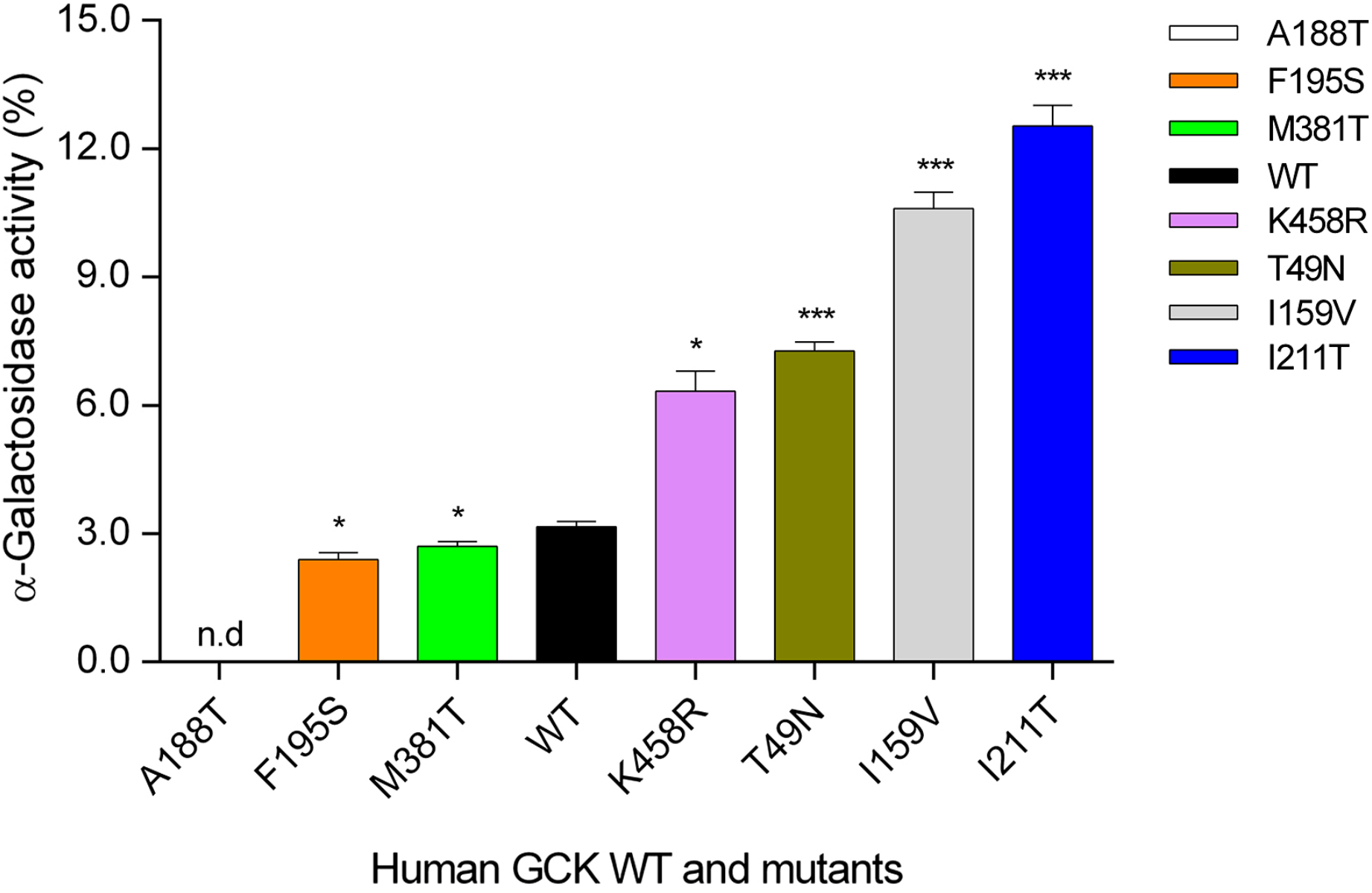
15.0
12.0
9.0
6.0
3.0
0.0
α-Galactosidase activity (%)
n.d
*
*
*
***
***
***
A188T
F195S
M381T
WT
K458R
T49N
I159V
I211T
Human GCK WT and mutants
A188T
F195S
M381T
WT
K458R
T49N
I159V
I211T

Figure 4

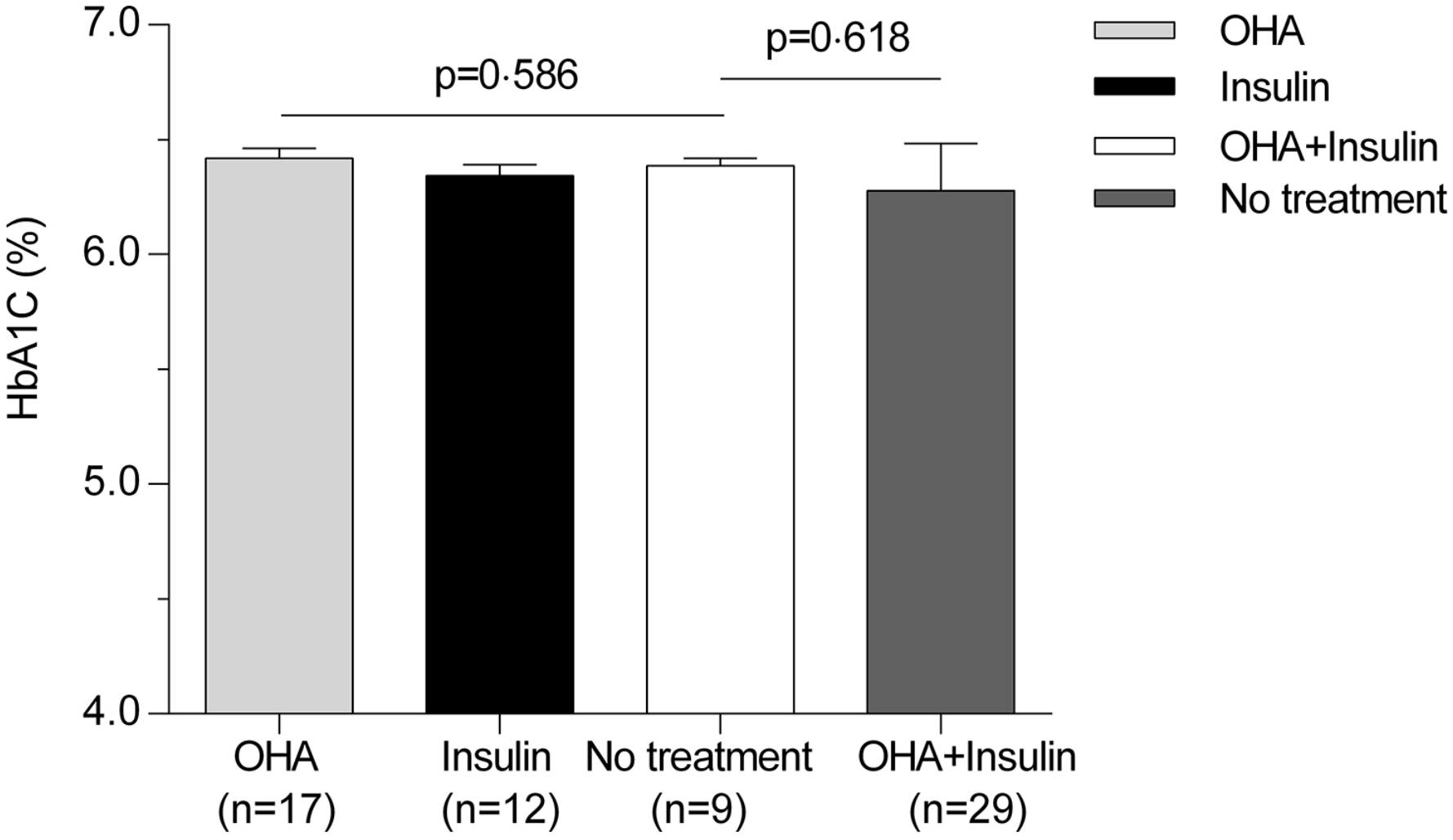
7.0
6.0
5.0
4.0
HbA1C (%)
p=0·586
p=0·618
OHA
Insulin
OHA+Insulin
No treatment
OHA
(n=17)
Insulin
(n=12)
No treatment
(n=9)
OHA+Insulin
(n=29)

Figure 5